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INSECT ENDOCHITINASES: GLYCOPROTEINS FROM MOULTING FLUID, INTEGUMENT AND PUPAL HAEMOLYMPH OF *MANDUCA SEXTA* L.*

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Abstract—Three endochitinases have been isolated from the tobacco hornworm, *Manduca sexta*, by ammonium sulphate fractionation and chromatographic procedures. The purified enzymes show single bands on sodium dodecyl sulphate acrylamide gel electrophoresis and have apparent mol. wts of 7.5×10^4 (I), 6.2×10^4 (II) and 5.0×10^4 (III). They are present in prepharate pupal integument, larval integument prior to apolysis, moulting fluid and pupal haemolymph. I and III are glycoproteins that contain glucosamine and several neutral hexoses. II was not examined for carbohydrate. The endochitinases cleave glycol chitin and chitin oligosaccharides ultimately to disaccharide and trisaccharide products. The longer substrates are preferred and no activity is expressed toward *p*-nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside, the β (1 → 4) linked dimer of *N*-acetylglucosamine or *Micrococcus lysodeikticus* cell walls. K_m and k_{cat} values for glycol chitin are 0.2 mg/ml and 2 sec^{-1}, respectively. The endochitinases are quite stable over a wide range of pH and exhibit maximum activity around pH 6 with the oligosaccharide substrates. Antibody raised against III cross reacts with II but not with I. The endochitinases in integument and moulting fluid probably digest chitin in old cuticle prior to moulting.

Key Words Index—Chitin, chitinase, moult, endochitinase, tobacco hornworm, haemolymph, glycoprotein, antibody, integument, moulting fluid, endochitinase

INTRODUCTION

β-*N*-ACETYLGLUCOSAMINIDASES are important carbohydrate-splitting enzymes whose substrates can be polysaccharides, oligosaccharides, glycoproteins, mucopolysaccharides or mucolipids. In many animals they are lysosomal enzymes whose deficiency leads to cellular pathology and clinical disease (NEUFELD *et al.*, 1975). In insects they are important enzymes involved with moulting whose excess or deficiency may lead to developmental failure (MUZZARELLI, 1977). The substrates for the insect enzymes are chitin, the primary structural polysaccharide in cuticle and peritrophic membrane, and oligosaccharides derived therefrom. Two specialized β-*N*-acetylglucosaminidases catalyze the catabolism of chitin in insects, endochitinase [endo-β(1 → 4) poly-*N*-acetylglucosaminidase] and exochitinase [exo-β(1 → 4)-oligo-*N*-acetylglucosaminidase]. The present report deals with the purification and characterization of endochitinases from the tobacco hornworm, *Manduca sexta* L. Properties of the exochitinases from *M. sexta* have been described previously (DZIADIK-TURNER *et al.*, 1981; KOGA *et al.*, 1982).

MATERIALS AND METHODS

Chemicals

Unless otherwise noted, all chemicals were of highest purity commercially available. 3,4-Dinitrophenyl-tetra-*N*-acetyl-β-D-chitotetraoside (BALLARDIE and CAPON, 1972) was from Research Products (Elk Grove, IL). *N*-Acetyl-[1-^{14}C]-glucosamine (250 m-Ci/m-mole) was from Amersham/Searle. Glycol chitin was prepared from crab chitin by glycolation with ethylene oxide followed by acetylation with acetic anhydride (SENZU and OKIMASU, 1950). *N*-Acetylglucosamine oligosaccharides were prepared from crab chitin by the method of RAFTERY *et al.* (1969) and were acetylated with acetic anhydride to ensure that no chitosan oligomers were generated. Elemental analyses for C, H, O and N were in accord with predicted values and purity was confirmed by HPLC (VAN EIKEREN and MCLAUGHLIN, 1977).

Insects

Eggs of *Manduca sexta* (L.) were a gift from the Metabolism and Radiation Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Fargo, ND. Lar-

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vae were reared on a standard diet (BELL and JOACHIM, 1976) and kept at 28°C and 60% r.h. on a 16 hr light–8 hr dark photoperiod. Animals were staged according to REINECKE *et al.* (1980).

Tissue collection

Moulting fluid and haemolymph were collected according to DZIADIK-TURNER *et al.* (1981). Integument was obtained after dissecting away as much extraneous tissue as possible from the epidermis and cuticle under saline (160 mM NaCl, 3 mM KCl, 1.8 mM $CaCl_2$, 0.2 mM Na_2HPO_4, 1.8 mM NaH_2PO_4, pH 7.2) mixed with 10^{-3} M diisopropylphosphorofluoridate (DFP)* and 10^{-5} M 1-phenyl-2-thiourea (PTU). Samples were used immediately or frozen at −20°C.

Endochitinase purification

Prepharate pupal cuticles were homogenized using a Tekmar tissumizer® in 20 mM Tris–HCl pH 7.8 containing 0.01 mM PTU, 1 mM DFP and 20% (w/v) sucrose (0.34 g tissue wet wt/ml) for 5 min. After centrifugation at 23,500 *g* for 30 min at 4°C, the particulate matter was homogenized at a second time and to the combined supernatant solutions ammonium sulphate was added to give 30% saturation. After recentrifugation at 23,500 *g* for 30 min at 4°C the supernatant was mixed with ammonium sulphate to give 70% saturation and stirred overnight. The precipitate was collected, dissolved in 50 mM sodium phosphate buffer pH 7.5 and dialyzed overnight against several litres of 5 mM phosphate pH 6.8. After recentrifugation as described above the supernatant was collected and applied to a hydroxylapatite column (2 cm i.d. × 20 cm) and elution was carried out with a gradient formed from 200 ml each of 5 mM and 90 mM sodium phosphate at pH 6.8 or pH 7.5. The chitinolytic fractions were finally subjected to gel filtration chromatography on a Biogel P-100 column (2 cm i.d. × 172 cm) in 50 mM ammonium bicarbonate pH 8.2.

Polyacrylamide gel electrophoresis

Electrophoresis was performed in slab gels at pH 8.5 to assess purity and mol. wt under denaturing conditions in 0.1% SDS buffer (WEBER *et al.*, 1972). After electrophoresis the gel was fixed in 5% (w/v) trichloroacetic acid and stained in 0.25% (w/v) Coomassie brilliant blue R-250 (Bio Rad Labs) dissolved in 45% methanol: 9% acetic acid: 46% H_2O. Destaining was achieved with several washes of 25% methanol: 7.5% acetic acid: 67.5% H_2O. For determination of mol. wt, phosphorylase (9.4×10^4), bovine serum albumin (6.8×10^4), ovalbumin (4.3×10^4), carbonic anhydrase (3.1×10^4), soybean trypsin inhibitor (2.15×10^4) and lysozyme (1.43×10^4) were used as standard proteins (Sigma).

Protein assay

Protein assays were performed using the method of LOWRY *et al.* (1951).

Hydrolysis of enzymes and amino acid and carbohydrate analyses

Purified enzymes were hydrolyzed for amino acid content in 6 M HCl containing 0.1% phenol according to MOORE and STEIN (1963). Amino acids were quantitated by the method of HILL *et al.* (1979) as *o*-phthaldialdehyde (*o*-PHTH) derivatives using high performance liquid chromatography (HPLC) on a Waters reverse phase C18 column. Neutral and amino monosaccharides were determined after acid hydrolysis (2 M HCl at 100°C for 2 hr in an evacuated sealed tube) on an anion-exchange column in 0.5 M borate, pH 8.6 as tritium-labeled alditols after reduction with [^{3}H]-sodium borohydride (Amersham Seale, 10 Ci/m-mole) (BARR and NORDIN, 1980). Retention times were compared with those of standard alditols.

Enzyme assay and kinetics

The enzymes were assayed for glycol chitin hydrolytic activity by following the production of reducing end groups colorimetrically with the potassium ferriferrocyanide reagent at 420 nm (IMOTO and YAGISHITA, 1971). The hydrolysis of β (1 → 4) linked oligomers of *N*-acetylglucosamine at 25°C in 50 mM sodium phosphate or a buffer composed of acetate, phosphate and borate salt (FRUGONI, 1957) was monitored by HPLC as described previously (DZIADIK-TURNER *et al.*, 1981; KOGA *et al.*, 1982). Kinetic parameters were determined by Lineweaver Burk plots and nonlinear least squares analyses as described previously (KOGA *et al.*, 1982). Error in individual determinations was < 10%, and all experimental points ($n = 5$–8) were used for the optimization method to determine k_{cat}, K_m and their s.e's by nonlinear programming. Hydrolytic activity using pNpGlcNAc was measured at 337 nm according to FORD *et al.* (1973). Lytic activity for *Micrococcus lysodeikticus* cell walls was measured turbidimetrically (NEUBERGER and WILSON, 1967). The hydrolysis of 3,4-dinitrophenyl-tetra-*N*-acetyl-β-D-chitotetraoside was monitored by both HPLC and spectrophotometrically at 400 nm (BALLARDIE and CAPON, 1972).

Preparation of antiserum and Ouchterlony assay

Antiserum was prepared by inoculating 2.3 kg New Zealand white albino rabbits intramuscularly in four sites with a total of 0.5 mg endochitinase III suspended in complete Freund's adjuvant. The animals were bled at the end of one week and each subsequent week thereafter. Serum was prepared as described previously (CAMPBELL *et al.*, 1970).

Ouchterlony gels were prepared in 1% (w/v) agarose, 50 mM sodium phosphate, pH 7.0 containing 0.85% sodium chloride and 0.01% thimerosol. Antigen and antiserum were placed in wells and allowed to incubate overnight at 25°C. Precipitation bands were visible within 12 hr. Other enzymes tested for crossreactivity included *Streptomyces chitinase* (Calbiochem, ICN Biochemical Co.), lysozyme (Worthington) and *M. sexta* exochitinases (DZIADIK-TURNER *et al.*, 1981).

RESULTS

Enzyme purification

The hydrolysis of glycol chitin was used to monitor the purification procedure for insect endochitinase. We found that commercially available glycol chitin was unsuitable as a substrate for *M. sexta* endochitinase, probably because the substrate was too highly modified (glycolated and deacetylated). Therefore we prepared samples of glycol chitin from crab chitin. The material was an excellent substrate and hydrolysis was linear over a 20-fold range of enzyme concentration, with glycol chitin concentrations from 0.02 to 0.5 mg/ml, at pH 4, 6 and 9 and with reaction times from 15–120 min.

Three separate enzymes were purified from *M. sexta* prepharate pupal integument by ammonium sulphate fractionation, hydroxylapatite chromatography and gel filtration procedures (Table 1). The enzymes were distinguishable by their chroma-

* *Abbreviations used:* DFP, diisopropylphosphorofluoridate; PTU, 1-phenyl-2-thiourea; SDS, sodium dodecyl sulfate; HPLC, high performance liquid chromatography; *o*-PHTH, *o*-phthaldialdehyde; pNpβGlcNAc, *p*-nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside; β (1 → 4) $GlcNAc_n$; β (1 → 4) linked oligosaccharides of *N*-acetylglucosamine.

Table 1. Purification of endochitinases I, II and III from *M. sexta* integument at prepharate pupal stage

Step	Total units*			Total protein (mg)			Specific activity (Unit/mg)			Overall yield (%)			Overall purification		
Starting material	3770†		8750‡	2300		3480	1.6		2.2	100		100	1		1
Ammonium sulphate fractionation	1690		4900	440		1120	3.8		4.3	44.8		56.1	2.3		2.0
Hydroxylapatite chromatography															
First	838		2627	17		67	48		39	22.2		30.0	29		18
Second	580		1915	4.6		17.6	127		109	15.6		21.9	77		50
Third	323		1000	2.3		8.3	138		120	8.6		11.4	84		55
	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
Gel filtration	174	37	595	1.1	0.2	4.0	165	168	149	4.6	1.0	6.8	101	102	68

* Unit = ΔA (0.02) at 420 nm after 1 hr at 32°C using 0.5 mg/ml glycol chitin in 50 mM sodium phosphate pH 6.4.
† Integument from 57 insects was used to isolate endochitinases I and II.
‡ Integument from 100 insects was used to isolate endochitinase III.

tographic and electrophoretic behaviour on hydroxylapatite and polyacrylamide gels. All three enzymes eluted together at 45 mM sodium phosphate pH 6.8 on hydroxylapatite. Upon rechromatography at pH 7.5, one of the endochitinases (III) eluted without adsorbing while the other two eluted at 45 mM buffer. Final purification was achieved by gel filtration chromatography on Biogel P-100. The endochitinases exhibited single bands after sodium dodecyl sulphate polyacrylamide gel electrophoresis (Fig. 1). Apparent mol. wts were estimated by comparison of mobilities to those of standard proteins to be 7.5×10^4, 6.2×10^4 and 5.0×10^4. The enzymes were denoted I, II and III based on their decreasing mol. wt and order of elution from gel filtration columns. The final purifications were 100-, 100- and 70-fold with overall

Table 2. Amino acid compositions of endochitinases I, II and III from *M. sexta*

Amino acid	Residues per molecule* I	II	III
Aspartic acid/ Asparagine	68.0 ± 1.9 (10.6)	54.0 ± 1.6 (10.1)	23.0 ± 0.7 (5.3)
Glutamic acid/	68.9 ± 0.3 (10.7)	46.0 ± 0.3 (8.6)	28.0 ± 0.2 (6.5)
Serine	35.3 ± 0.2 (5.5)	29.7 ± 0.9 (5.5)	29.5 ± 0.1 (6.8)
Histidine	17.7 ± 0.3 (2.8)	13.3 ± 0.2 (2.5)	5.6 ± 0.1 (1.3)
Threonine	38.5 ± 0.8 (6.0)	35.6 ± 0.7 (6.6)	21.1 ± 0.4 (4.9)
Glycine	51.9 ± 1.7 (8.1)	44.5 ± 1.6 (8.3)	43.5 ± 1.6 (10.0)
Alanine	39.4 ± 1.2 (6.1)	33.6 ± 1.0 (6.3)	28.3 ± 0.8 (6.5)
Tyrosine	26.4 ± 0.1 (4.1)	25.3 ± 0.1 (4.7)	31.4 ± 0.1 (7.2)
Arginine	33.6 ± 2.0 (5.2)	21.8 ± 1.3 (4.1)	20.2 ± 1.3 (4.7)
Valine	43.4 ± 0.4 (6.8)	33.9 ± 0.3 (6.3)	31.5 ± 0.2 (7.3)
Methionine	3.9 ± 0.1 (0.6)	8.7 ± 0.1 (1.6)	7.4 ± 0.1 (1.7)
Isoleucine	25.6 ± 0.1 (4.0)	22.8 ± 0.1 (4.2)	21.5 ± 0.1 (5.0)
Leucine	38.7 ± 0.6 (6.0)	35.5 ± 0.4 (6.6)	33.9 ± 0.4 (7.8)
Phenylalanine	23.9 ± 0.4 (3.7)	21.6 ± 0.3 (4.0)	21.0 ± 0.3 (4.9)
Lysine	44.4 ± 2.1 (6.9)	41.3 ± 1.9 (7.7)	32.0 ± 1.4 (7.4)
Tryptophan	25 (3.9)	21 (3.8)	17 (3.9)
Proline	n.d. (5.5)	n.d. (5.5)	n.d. (5.3)
Cysteine	n.d. (3.4)	n.d. (3.5)	n.d. (3.5)
Total residues	585	489	395
Glucosamine	~4	n.d.	~3
Neutral hexose	~7	n.d.	~6
Molecular weight	75000	62000	50000

* Mean values ± S.E. from three analyses of protein hydrolyzed for 24 hr *in vacuo* in 6M HCl containing 1% phenol. Mole percentage given in parentheses. Proline, cysteine and tryptophan not quantitated by *o*-PHTH-HPLC method. For the purpose of comparison, values for proline and cysteine assumed from average percentage composition of amino acids in 108 proteins (DAYHOFF and HUNT, 1972). Tryptophan estimated from molar extinction coefficients at 280 nm and amounts of tyrosine and phenylalanine determined by amino acid analyses.

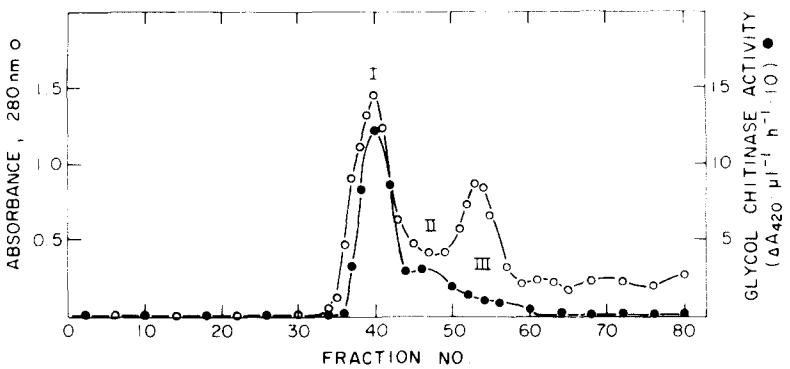

Fig. 2. Gel filtration chromatography of pharate pupal moulting fluid from *M. sexta*. Lyophilized fluid was reconstituted in 50 mM ammonium bicarbonate pH 8.2 containing 10^{-3} M diisopropylphosphorofluoridate and passed over a 2 × 172 cm Biogel P-100 column at 4°C. Symbols: absorbance at 280 nm, ○——○; glycol chitin hydrolytic activity, ●—●. Endochitinase I eluted in fractions 37–43, II in 45–50 and III in 51–57.

yields of 5, 1 and 7%. The specific activities of the three endochitinases were quite similar (149–168 units of activity per milligram protein, Table 1).

Chemical composition

The amino acid and carbohydrate compositions of the endochitinases are listed in Table 2. Enzymes I and III are glycoproteins and contained about 2–3% carbohydrate. We did not perform a carbohydrate analysis of enzyme II due to the low amount of protein purified but it seems reasonable to assume that it also is glycosylated to about the same extent. The carbohydrate was composed of both hexosamine (1% *N*-acetylglucosamine) and small quantities of neutral hexoses (<1% fucose, galactose, mannose, glucose and rhamnose) which indicates the presence of unique heteropolysaccharide side chains of a rather complex type. The endochitinases also exhibited different amino acid compositions.

Tissue distribution of endochitinases

We utilized gel filtration chromatography to determine which endochitinase, if any, is present in other tissues of *M. sexta*. Pharate pupal moulting fluid contained a high level of endochitinase I and smaller amounts of II and III (Fig. 2). Like prepharate pupal integument, extracts of larval integument obtained from animals just prior to apolysis exhibited all three enzymes but in relative amounts that varied considerably. One preparation contained primarily endochitinase I and another endochitinase III. The chitinases were also present in pupal haemolymph but larval haemolymph was devoid of activity.

pH stability and dependence

The stability of one of the endochitinases, III, was examined after incubation at pH 2.3–10.9 at 25°C (Fig. 3). The enzyme (52 nM) was stable for up to 12 hr between pH 6.5 and 10 (≥80% active). The two other endochitinases were also quite stable over a wide range of pH. This stability is important for enzymes that participate in moulting, a process which apparently requires a number of hours to complete. Whether the endochitinases are as stable *in vivo* is unknown.

The effect of pH on endochitinase activity was also examined using glycol chitin as substrate (Fig. 4). All three enzymes exhibited an unusual profile that indicated two pH optima, one at pH 5–6 and the other pH 8.5–10. This unusual kinetic behaviour may be

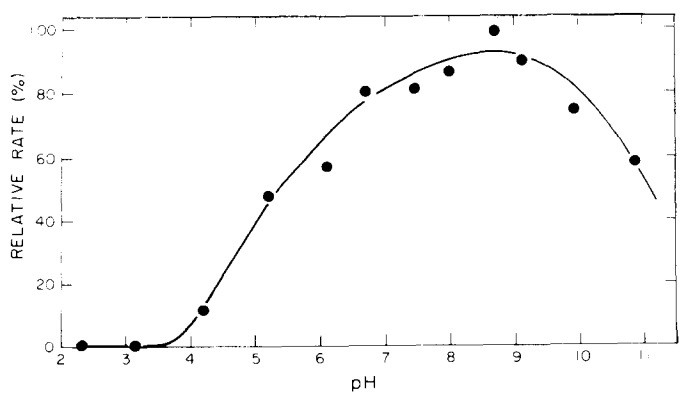

Fig. 3. Effect of preincubation pH on endochitinase III. Enzyme solution was incubated for 12 hr at 25°C pH 2.3–10.9 and assayed with glycol chitin (0.5 mg/ml) in 50 mM sodium phosphate, pH 6.4 for 1 hr at 32°C. Mean values from two determinations with S.E. ≤ 5% are shown.

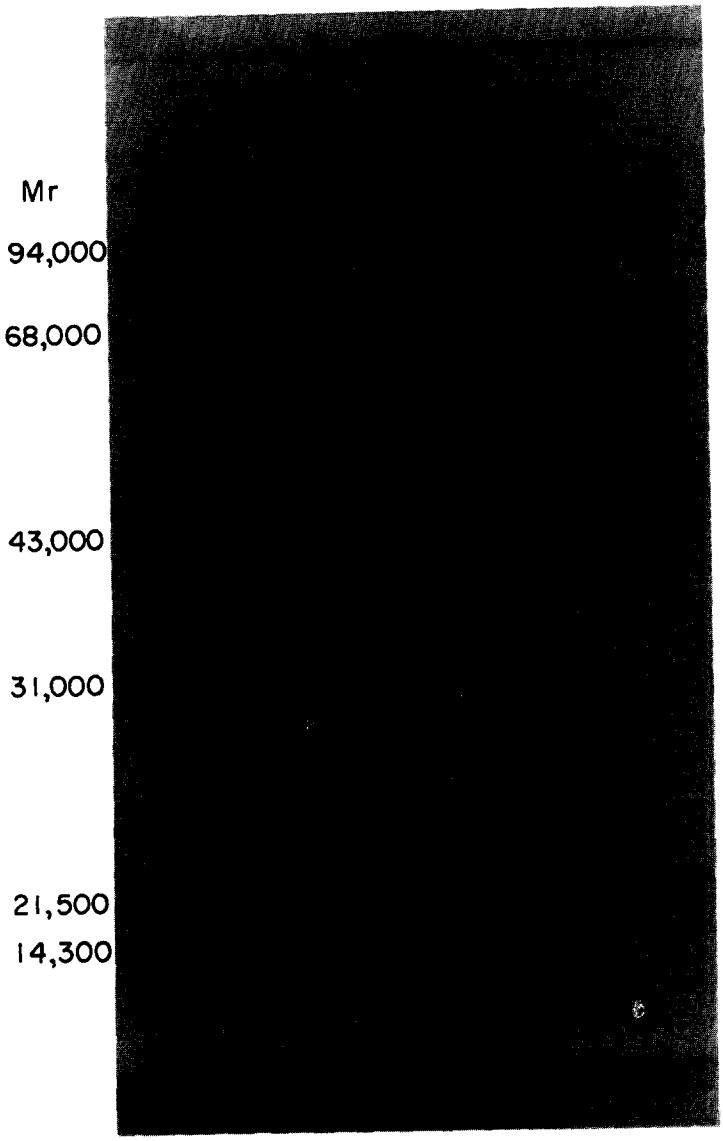

Fig. 1. SDS polyacrylamide slab gel electrophoresis of *M. sexta* endochitinases. Track A: phosphorylase A, 9.4×10^4; bovine serum albumin, 6.8×10^4; ovalbumin, 4.3×10^4; carbonic anhydrase, 3.1×10^4; soybean trypsin inhibitor, 2.15×10^4; lysozyme 1.43×10^4. B: endochitinase III. C: endochitinase II. D: endochitinase I.

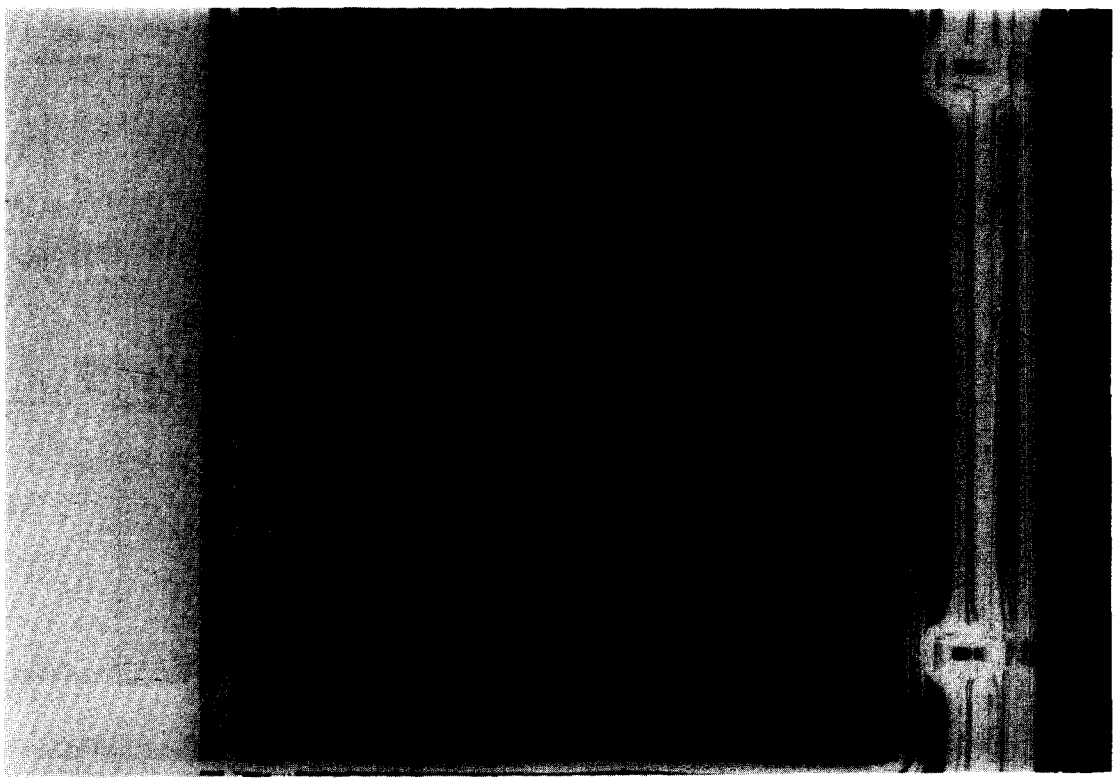

Fig. 8. Immunodiffusion analysis of *M. sexta* endochitinases with antiserum to endochitinase III. The centre well received 15 μl of antiserum directed against endochitinase III. The peripheral wells contained about 0.5 μg in 15 μl of endochitinase III (1), II (2) and I (3). The other wells were not utilized.

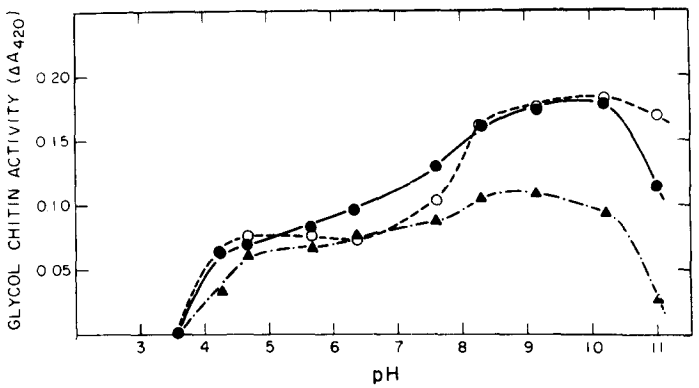

Fig. 4. Effect of pH on the degradation of glycol chitin by *M. sexta* endochitinases. Symbols: endochitinase I ●—●, II, ○—○; III, ▲—▲. Enzyme solutions (≃30 nM) were mixed with glycol chitin (0.1 mg/ml) in universal buffer (pH 3.55 to 11.0) for 1 hr at 32°C. Mean values from two or three determinations with S.E. ≤ 10% are shown.

due to the presence of different substrate forms that either vary in polymer chain length or are partially deacetylated and thus composed of a small proportion of glucosamine. With regard to the latter, deprotonation of the amino group of glucosamine ($pK_a \simeq 7.8$) would deionize the substrate and probably facilitate substrate binding and hydrolysis. We also used a small tetrasaccharide substrate, $\beta(1 \rightarrow 4)$ $GlcNAc_4$, to study pH effects. This substrate and its hydrolysis product are less complex than glycol chitin and are less susceptible to unusual pH effects. Only one glycosidic bond is hydrolyzed and one product, $\beta(1 \rightarrow 4)GlcNAa_2$ (dimer), is generated (Fig. 5). The pH activity curves from tetramer showed a single pH optimum between pH 5–7 and a rather asymmetrical shape (Fig. 6). Fifty percent of maximum activity occurred at approx. pH 4 on the acid side of the profile and at pH 9 on the alkaline side. This result suggested that an ionized acidic group and one or more protonated basic groups are required for enzymatic activity in all three enzymes.

Kinetic parameters and substrate specificity

Analysis of initial velocity experiments with glycol chitin as substrate yielded the kinetic parameters listed in Table 3. For all endochitinases at pH 4.3, 6 and 9 the K_m values were essentially identical (≃0.2 mg/ml). Values for k_{cat} were more varied. At pH 6 and 9, k_{cat} values were similar for enzymes I and III ($\simeq 1\ sec^{-1}$). III exhibited a three-fold lower k_{cat} value at pH 4.3. At pH 9 endochitinase II has a two-fold higher k_{cat} value. Upward curvature of Lineweaver–Burk plots suggested that substrate inhibition occurred at glycol chitin concentrations which depend on pH and the form of endochitinase utilized (Fig. 7A). II and III were enzymes most and least susceptible to substrate inhibition at pH 9 and 4.3 (Table 3). Chitin oligosaccharides exhibited even more atypical reciprocal plots and were hydrolyzed faster at lower concentrations than at higher ones (Fig. 7B). The simplest interpretation of these results was that all of the substrates tested, especially the smaller ones, are cap-

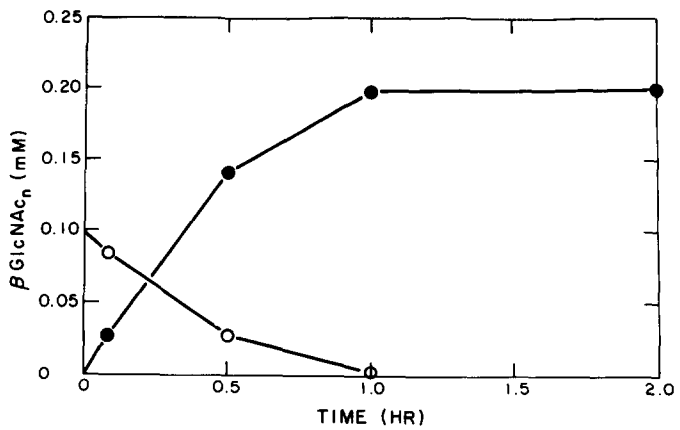

Fig. 5. Time course for the hydrolysis of β $(1 \rightarrow 4)$ linked tetramer of *N*-acetylglucosamine by *M. sexta* endochitinase III. Enzyme (18 nM) was mixed with $\beta(1 \rightarrow 4)$ $GlcNAc_4$ (0.9 mM) in 50 mM sodium phosphate pH 5.3 at 25°C. Aliquots were taken at various times and reaction was stopped by adjustment of pH to 2.5 with phosphoric acid. Symbols: β $(1 \rightarrow 4)$ $GlcNAc_4$, ○—○; β $(1 \rightarrow 4)$ $GlcNAc_2$, ●—●. Typical data from one experiment are presented with S.E.M. ≤10%.

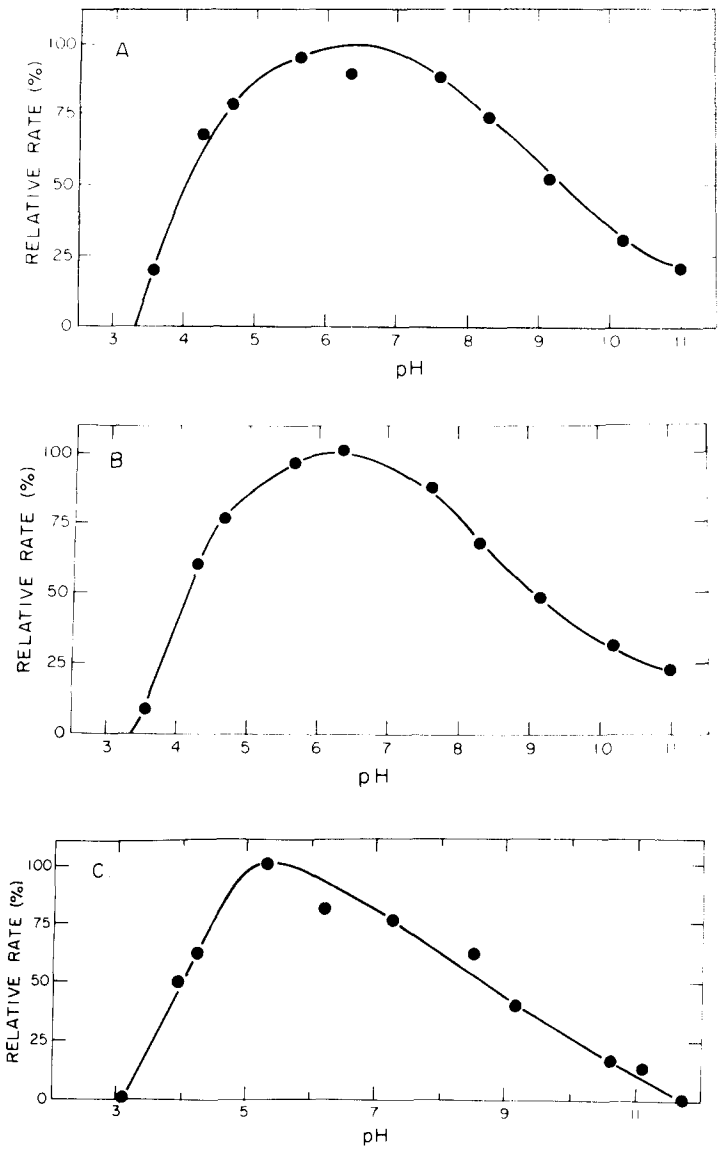

Fig. 6. Effect of pH on the hydrolysis of $\beta(1 \rightarrow 4)$ $GlcNAc_4$ by endochitinases from *M. sexta*. Homogeneous enzymes (15 nM) were mixed with tetramer (0.09 mM) at 25 C. Reaction was quenched after 20 min by addition of dilute H_3PO_4 to pH 2.5. A: endochitinase I, B: endochitinase II. C: endochitinase III. Shown are mean values from two determinations with S.E. ≤ 10.

Table 3. Kinetic parameters for hydrolysis of glycol chitin by *M. sexta* endochitinases I, II and III

Enzyme	pH*	$k_{cat}(sec^{-1})$†	K_m (mg/ml)†	k_{cat}/K_m	Substrate inhibition (mg/ml)
I	6.0	1.15 ± 0.28	0.14 ± 0.05	8.2	>0.12
	9.0	1.30 ± 0.17	0.15 ± 0.03	8.7	>0.12
II	9.0	3.08	0.23	13.4	>0.05
III	4.3	0.56 ± 0.05	0.20 ± 0.04	2.8	>1.0
	6.0	1.56 ± 0.40	0.20 ± 0.07	7.8	>0.12
	9.0	1.68 ± 0.40	0.23 ± 0.07	7.3	>0.12

* Universal buffer ($\mu = 0.1$) was used at pH 4.3 and 9.0. Sodium phosphate (50 mM) was used at pH 6.0.

† $S_0 = 0.02$–0.5 mg/ml. $E_0 = 18$–38 nM. Mean values for 5–8 determinations ± S.E.

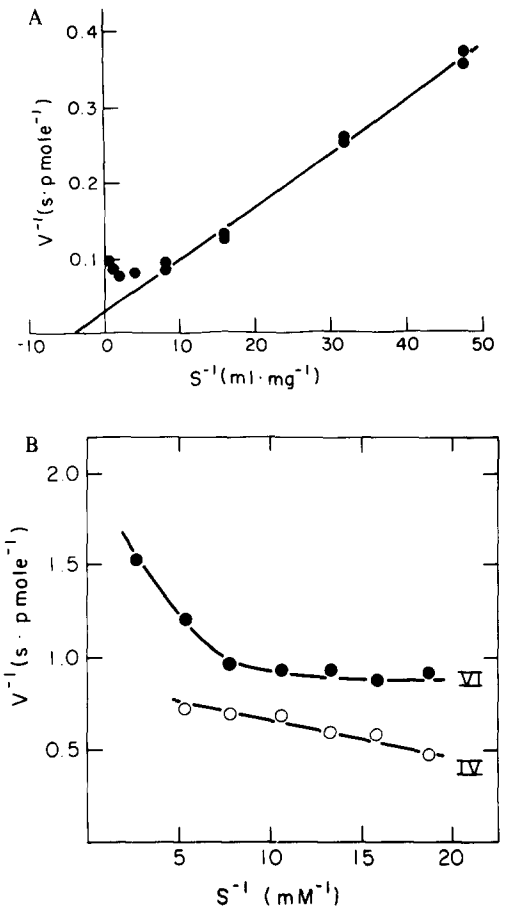

Fig. 7. Reciprocal plots for the hydrolysis of glycol chitin and chitin hexasaccharide by *M. sexta* endochitinase III. A. Glycol chitin was incubated with enzyme (28 nM) in 50 mM sodium phosphate buffer pH 6.0 at 25°C for 2 hr. B. Oligosaccharide was mixed with endochitinase III (20 nM) in pH 5.3 buffer at 25°C. Hydrolysis was stopped after 15 min by adjustment of pH to 2.5 (KOGA *et al.*, 1982).

able of enzyme inhibition. Apparently, multiple modes of binding to endochitinases occur with polymeric and oligometric substrates including nonproductive ones. Exochitinases from *M. sexta* were also susceptible to substrate inhibition (DZIADIK-TURNER *et al.*, 1981; KOGA *et al.*, 1982).

The high degree of substrate inhibition of the endochitinases by the chitin oligosaccharides prevented an absolute determination of K_m and k_{cat} with these substrates. However, a relative comparison of the initial rates and cleavage patterns of oligosaccharide and glycol chitin substrates by the three enzymes under identical conditions is given in Table 4. I, II and III behaved primarily as endochitinases with II the most active followed by III and I. The rates of hydrolysis increased with length of substrate. No cleavage of disaccharide occurred and glycol chitin was degraded at a rate of at least five-fold faster than the *N*-acetylglucosamine oligomers. Oligosaccharides were degraded ultimately to disaccharide and/or trisaccharide, after which slow hydrolysis of the latter occurred. The time course for hydrolysis of tetrasaccharide revealed chitobiose as the sole product (Fig. 5). Pentasaccharide was hydrolyzed to dimer and trimer while hexasaccharide also yielded chitotriose as the initial product. Thus, the endochitinases expressed a slight exoenzyme activity, but only with chitotriose as substrate. A nitrophenylated substrate, 3,4-dinitrophenyltetra-*N*-acetyl-β-D-chitotetraoside, was also utilized to determine the cleavage pattern of the endochitinases. HPLC and spectrophotometric analyses revealed that no *N*-acetylglucosamine or 3,4-dinitrophenol was produced during the catalysis; only trisaccharide, disaccharide and dinitrophenylated oligosaccharides were produced. All of these results were consistent with a mechanism where the enzymes cleave off oligosaccharides from chitin following an endoenzyme pattern.

The endochitinases exhibited no activity toward *p*-nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside, a typical substrate for exochitinases (FORD *et al.*, 1973; KOGA *et al.*, 1982). No lysozyme-like activity was detected when the enzymes were tested using *Micrococcus lysodeikticus* cell walls as substrate. The assays used endochitinase concentrations that were 1000 times higher than the minimal detectable level of *M. sexta* exochitinase or hen's egg white lysozyme. When endochitinase III was preincubated with calcium ion (10^{-3} M), ethylene dinitrilotetraacetic acid (10^{-4} M) or DFP (10^{-3} M), there was no effect on

Table 4. Rates and cleavage patterns of substrate hydrolysis by *M. sexta* endochitinases I, II and III

Substrate	Velocity (p-mole/sec)* I	II	III	Cleavage pattern
β(1→4)GlcNAc$_2$	0	0	0	$X_1 - X_2$
β(1→4)GlcNAc$_3$	0.62 ± 0.08 [5]† (51)	1.21 ± 0.15 [4] (100)	0.89 ± 0.12 [6] (73)	↓ or ↓ $X_1 - X_2 - X_3$
β(1→4)GlcNAc$_4$	1.26 ± 0.01 [10] (40)	3.18 ± 0.15 [11] (100)	1.90 ± 0.01 [12] (60)	↓ $X_1 - X_2 - X_3 - X_4$
β(1→4)GlcNAc$_5$	2.01 ± 0.05 [17] (53)	3.81 ± 0.23 [13] (100)	3.21 ± 0.04 [21] (84)	↓ or ↓ $X_1 - X_2 - X_3 - X_4 - X_5$
Glycol chitin	12.02 ± 1.54 [100] (42)	28.53 [100] (100)	15.58 ± 3.74 [100] (55)	↓ ↓ $X_1 - X_2 - X_3 - - X_{n-1} - X_n$

* $E_0 = 18 \times 10^{-9}$ M; oligosaccharide concentration (S_0) = 94×10^{-6} M; glycol chitin concentration (S_0) = 0.02–0.1 mg/ml; 50 mM sodium phosphate, pH 5.3, 25°C. Mean values from three determinations ± S.E.

† Number in bracket gives relative rate of hydrolysis of all substrates by one enzyme.

‡ Number in parenthesis gives relative rate of hydrolysis of one substrate by all enzymes.

activity when assayed using either glycol chitin or an oligosaccharide as substrate.

When $\beta(1 \rightarrow 4)$ $GlcNAc_6$ (hexamer, 0.09 mM) was hydrolyzed by endochitinases I, II or III (17 nM) in the presence of *N*-acetyl-[1-^{14}C]-glucosamine (1.1 mM, 5 μCi), no radioactivity was incorporated into the oligosaccharide products separated by HPLC after 40% extent of hydrolysis. Thus, the endochitinases did not catalyze glycosyl transfer to *N*-acetylglucosamine and transglycosylation was not a property of these enzymes.

Immunological characterization

Antibody to endochitinase III (Ab III) was produced in rabbits and its reactivity with the three endochitinases was examined by the Ouchterlony double-diffusion procedure. Antisera to endochitinases I and II were not elicited because of inadequate supplies of homogeneous enzymes. Ab III cross-reacted with endochitinase II but not with I (Fig. 8). The similar precipitin lines, together with their fused pattern, suggested that II and III are closely related antigenically. Endochitinase I appeared to be unrelated. Ab III did not cross react with commercially available chitinases from Streptomyces species, hen's egg white lysozyme or with two exochitinases from *M. sexta* (DZIADIK-TURNER *et al.*, 1981). Conversely, antisera to the exochitinases failed to cross react with any of the endochitinases from the same species (KOGA *et al.*, 1983).

DISCUSSION

Although the importance of chitinolytic enzymes in the growth and development of insects has been recognized for many years, little work has been published on the properties of purified enzymes (MUZZARELLI, 1977). This is, to our knowledge, one of the first reports on the characterization of homogeneous endochitinases from insect integument. We have purified and characterized three endochitinases from tissues of the tobacco hornworm. The low overall purification values (70–100-fold) suggest that these enzymes comprise a significant percentage of the total protein present in the tissues analyzed. The endochitinases differed in size, amino acid content and carbohydrate composition. All three were present in integument from larvae and pharate pupae that are beginning to moult, in moulting fluid and in pharate pupal haemolymph but not in larval haemolymph. Immunologically, two of the three enzymes were related. The insect endochitinases exhibited normal kinetic behaviour and had similar kinetic properties. They were very efficient at hydrolyzing polymeric and oligomeric substrates composed of $\beta(1 \rightarrow 4)$ linked *N*-acetylglucosamine units via an endoenzyme cleavage pattern. When presented with large substrates, the enzymes preferentially liberated oligosaccharides with a degree of polymerization of 3 or greater. Their kinetic parameters differed only by a factor of two or less. They were susceptible to substrate inhibition but that was not unusual for enzymes which modify polymeric substrates. Their active sites may be large open clefts composed of several subsites that accommodate both productive and nonproductive modes of binding. BADE and STINSON (1979) have attributed curved kinetics of a heterogeneous chitinase preparation from *M. sexta* to allosteric behaviour of the enzyme. We did not observe such kinetic behaviour with homogeneous endochitinases.

Endochitinases from various sources differ widely in their physical and kinetic properties. Insect endochitinases occur in several tissues and in assorted sizes. Mammalian sera contain enzymes that are about the same size, $5–6 \times 10^4$ (LUNDBLAD *et al.*, 1974, 1979), while plant endochitinases are smaller, 3×10^4 (MOLANO *et al.*, 1979). The pH optima appear to depend on the enzyme source and substrate used. Low optima ($\simeq$pH 2) were observed with yeast, goat and bovine endochitinases when glycol chitin or radiolabelled chitin was the substrate (LUNDBLAD *et al.*, 1974, 1979; CORREA *et al.*, 1982). However, the latter two enzymes showed maximum activity with colloidal chitin at the same pH as that found for the *M. sexta* enzymes (pH 6). Fungal and plant endochitinases also had pH optima of 6 (MOLANO *et al.*, 1979; BERGER and REYNOLDS, 1958). BADE and STINSON (1981) reported that chitinolytic activity in *M. sexta* moulting fluid is maximal at a slightly higher pH, 7, but that it is more reproducible at pH 6.5. They used raw moulting fluid and insoluble chitin in their experiments, however. Similar K_m values were exhibited for glycol chitin by the mammalian and insect enzymes.

KIMURA (1974, 1976) reported previously that endochitinases similar to those of *M. sexta* are present in the silkworm, *Bombyx mori* L. during larval and pupal development. Endochitinases were detected in integument, moulting fluid and several other tissues. Two peaks of activity were separated from moulting fluid by gel filtration.

Chitinolytic enzymes have also been partially characterized from integument of *Drosophila hydei* (SPINDLER, 1976) and *Locusta migratoria* (ZIELKOWSKI and SPINDLER, 1978). Like the result with hornworm integument, gel filtration separated three endochitinase fractions from *Drosophila* integument. The number of locust endochitinases was not determined. The pH optima of these endochitinases occurred at about pH 5. In all of these species, enzymes reached their highest levels during the latter part of the moulting cycle and declined shortly before ecdysis.

Chitinolytic activity also occurs in gut tissues from arthropods. An endochitinase was purified from the digestive fluid of the spider, *Cupiennius salei* (MOMMSEN, 1980). It had an apparent mol. wt of 5×10^4 and a pH optimum 7. This enzyme probably digests carbohydrate food sources containing chitin.

The endochitinases in *M. sexta* may have varied functions depending on their tissue localization. In integument and moulting fluid they probably digest chitinous structures that are discarded during a moult. They generate oligosaccharides that are further degraded to *N*-acetyl-glucosamine by exochitinases (DZIADIK-TURNER *et al.*, 1981; KOGA *et al.*, 1982). In haemolymph they may catabolize glycolipid or glycoprotein substrates and be related to endo-β-*N*-acetylglucosaminidases found in sera from higher animals (LEABACK 1970; NEUFELD *et al.*, 1975). It is interesting to note that the insect endochitinases are themselves glycoproteins which contain *N*-acetylglucosamine and several neutral hexoses. Apparently yeast endochitin-

ase is also a glycoprotein with mannan as the carbohydrate moiety (CORREA *et al.*, 1982) while wheat germ endochitinase is not glycosylated (MOLANO *et al.*, 1979).

We have purified and characterized two types of chitinolytic enzymes from the tobacco hornworm. During the moulting cycle these enzymes are probably responsible for the total depolymerization of chitin in cuticle. Endochitinases produce oligosaccharides that in turn are hydrolyzed to monomer by exochitinases. Inhibition of the chitinases or β-*N*-acetylglucosaminidases would be detrimental to moulting, digestion and certainly other metabolic processes. A determination of how these enzymes are regulated *in vivo* and *in vitro* and also of how they can be inhibited or activated may lead to new developments in insect control.

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